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The Host as the Driver of the Microbiota in the Gut and External Environment of *Drosophila melanogaster*

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Most associations between animals and their gut microbiota are dynamic, involving sustained transfer of food-associated microbial cells into the gut and shedding of microorganisms into the external environment with feces, but the interacting effects of host and microbial factors on the composition of the internal and external microbial communities are poorly understood. This study on laboratory cultures of the fruit fly *Drosophila melanogaster* reared in continuous contact with their food revealed time-dependent changes of the microbial communities in the food that were strongly influenced by the presence and abundance of *Drosophila*. When germfree *Drosophila* eggs were aseptically added to nonsterile food, the microbiota in the food and flies converged to a composition dramatically different from that in fly-free food, showing that *Drosophila* has microbiota-independent effects on the food microbiota. The microbiota in both the flies that developed from unmanipulated eggs (bearing microorganisms) and the associated food was dominated by the bacteria most abundant on the eggs, demonstrating effective vertical transmission via surface contamination of eggs. Food coinoculated with a four-species defined bacterial community of *Acetobacter* and *Lactobacillus* species revealed the progressive elimination of *Lactobacillus* from the food bearing few or no *Drosophila*, indicating the presence of antagonistic interactions between *Acetobacter* and *Lactobacillus*. *Drosophila* at high densities ameliorated the *Acetobacter/Lactobacillus* antagonism, enabling *Lactobacillus* to persist. This study with *Drosophila* demonstrates how animals can have major, coordinated effects on the composition of microbial communities in the gut and immediate environment.

From a microbiological perspective, an animal is a transient, nutrient-rich patch. The capacity of various microorganisms to exploit the animal habitat involves multiple traits, including mechanisms that evade or modulate the animal immune system (1–3) and metabolic adaptations to utilize host resources (4, 5). Animal-associated microorganisms include pathogens, whose fitness is coupled to host disease and debility, and beneficial forms that variously contribute nutrients, confer protection, and deliver effectors that promote host performance (6). Consequently, the composition of animal-associated microorganisms is an important determinant of animal fitness.

Many animal-microbe associations are open systems, meaning that external microorganisms have access to the host habitat and members of the host microbiota are released back to the external environment via feces, sloughed skin, fluid secretions, etc. (7). Open symbioses can be invaded by external microorganisms that are compatible with the host and are competitive with resident microbiota. As a result, the host is potentially more exposed to parasites and cheats than in a closed system but also has an enhanced capacity to modify the composition of its microbiota adaptively to changes in environmental circumstances (8, 9). The shedding of microbial cells from hosts can alter the composition and functional traits of the microbial communities in the immediate environment, provide a source of compatible microbial inocula for other host individuals, including progeny, and promote dispersal of animal-associated microorganisms (10–13). These multiple studies indicate that the microbial communities in the external environment can both influence, and be influenced by, the microbiota in animals, but the scale of these reciprocal effects is largely unknown.

The purpose of this study was to identify the driver(s) of the microbial community composition in the linked host and external environment of an open symbiosis. The experiments were conducted on the association between *Drosophila melanogaster* fruit flies and their gut microbiota. Natural populations of *D. melanogaster* consume microorganisms associated with rotting fruit, including various yeasts and bacteria of the *Acetobacteraceae* and *Lactobacillales* taxa, and the flies mediate the dispersal of the bacteria via their feces (14–17). In laboratory culture, all life stages of *Drosophila* (eggs, larvae, pupae, and adults) are cohoused in vials of agar-based food, enabling precise quantification and manipulation of the microorganisms that cycle between the food and the *Drosophila* hosts (18–21).

The specific goal of this study was to determine how the abundance and composition of the microbiota are influenced by, first, microbial interactions with the food and with *Drosophila* and, second, microbe-microbe interactions. Our experiments compared the composition of the bacterial communities in the two habitats (flies and food) over the course of fly development and used *Drosophila* organisms colonized with defined sets of bacteria to investigate the processes by which the microbial community in

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one habitat can alter the community composition of the other habitat.

MATERIALS AND METHODS

Flies and bacteria. *Wolbachia*-free *Drosophila melanogaster* strain Canton S was maintained at 25°C under a 12-h:12-h light:dark cycle on yeast-glucose food obtained from the Cornell *Drosophila* Media Kitchen, comprising 100 g glucose liter^{-1} (Sigma, St. Louis, MO, USA), 100 g liter^{-1} inactive yeast (MP Biomedicals, Santa Ana, CA, USA), 12 g agar liter^{-1} (MP Biomedicals), and preservatives (0.04% phosphoric acid, 0.42% propionic acid; Sigma).

Experiments were initiated with eggs deposited overnight by mated females. Conventional *Drosophila* flies were derived from unmanipulated eggs rinsed briefly in sterile water. Axenic *Drosophila* flies were obtained by dechorionating eggs in 0.6% sodium hypochlorite, as described previously (22); the bacteria-free condition of dechorionated eggs was confirmed by negative results of PCR amplification with general bacterial primers and the absence of bacterial growth from eggs transferred to standard medium (methods described below). Gnotobiotic *Drosophila* flies were generated by adding dechorionated eggs to each vial of food, followed by 50 μl bacterial suspension at 10^8 cells ml^{-1} (5×10^6 cells per vial). The administered bacteria were clonal isolates of *Acetobacter pomorum* DmelCS_004, *Acetobater tropicalis* DmelCS_006, *Lactobacillus brevis* DmelCS_003, and *Lactobacillus plantarum* DmelCS_001, derived originally from guts of *D. melanogaster* Canton S flies (21) and grown overnight at 30°C in liquid modified MRS (MMRS) medium containing the following ingredients (from Sigma, unless stated otherwise): 1.25% vegetable peptone (Becton Dickinson), 0.75% yeast extract, 2% glucose, 0.5% sodium acetate, 0.2% dipotassium hydrogen phosphate, 0.2% triammonium citrate, 0.02% magnesium sulfate heptahydrate, 0.005% manganese sulfate tetrahydrate, 1.2% agar (Apex). The bacteria were administered to the dechorionated eggs either individually at 5×10^6 cells per vial or as a four-species inoculum, with each bacterial species provided at 1.25×10^6 cells per vial.

The experiments that included food sampling were conducted using flat-bottomed vials (2-cm internal diameter by 9-cm height) containing 7.5 g of food. Each food sample was collected into a sterile 1-ml pipette tip and then expelled into a previously weighed, sterile 1.5-ml centrifuge tube, which was then reweighed to obtain the weight of the food sample. A single sample was taken per vial: either a "surface sample" taken across the top 1 to 2 mm of the food or a "core sample" obtained by inserting the tip vertically to the bottom of the vial. All fly samples used for bacterial quantification by pyrosequencing, quantitative PCR (qPCR), and CFU counts were rinsed with sterile phosphate-buffered saline (PBS) prior to analysis.

Pyrosequencing analyses. DNA was extracted from each *Drosophila* fly, food sample, and environmental sample (swabs of fly incubator), along with a reagent-only control, by the salting-out method (23, 24). 16S rRNA gene amplicons were prepared using the general primer pair 27F-338R tagged with different multiplex identifiers (MIDs) (Table 1), as previously described (21, 24). Equal amounts (in nanogram quantities) of triplicate PCR products were mixed and purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), followed by quantification using PicoGreen. Emulsion PCR was conducted at 1.5 copies per bead using only the "A" beads for unidirectional 454 GS-FLX pyrosequencing with standard titanium chemistry.

Pyrosequencing flowgrams were analyzed using QIIME 1.7.0 virtualbox software, as described in reference 21, with default parameters (25), except the denoising cutoff was set to remove singletons. Taxonomy assignment of the operational taxonomic units (OTUs) clustered at 97% identity was conducted based on phylum-to-genus identities obtained by using the RDP classifier in QIIME trained on the Greengene reference database (May 2013), with additional information on species identities of the OTUs derived from NCBI StandAlone BLAST (the megaBLAST program is available at www.ncbi.nlm.nih.gov/blast/html/megablast.html), using the 16S rRNA gene microbial database (March 2015), which also provides the percent sequence identity to the top hit, confidence bit score, and E value. Supplementary manual curation was applied that removed a single OTU annotated as chloroplasts and to correct species identities of a few OTUs guided by data obtained from full-length 16S rRNA gene sequences in previous studies (see footnotes for Table S1 in the supplemental material). OTUs with fewer reads than in the reagent-only control were excluded. To minimize inflation of diversity due to sequencing errors, the presence of artifactual OTUs that can be explained by sequencing errors was assessed by a modified version of the Poisson probability method used previously (26). Briefly, the Poisson probabilities (PPois) for the occurrence of a single artifactual read at different sequence percent identities were calculated for each biological sample, based on a sequencing error rate of 1.07% (27). The probability of a minor OTU arising from sequencing errors was determined by normalizing the PPois to the power of the read number of that OTU in a given biological sample (PPois read number of the OTU in the sample). OTUs classified as artifacts were pooled with major OTUs with the same BLAST top hit. Principal coordinates analysis (PCoA) plots of the bacterial communities were created with jackknifing (rarified to the lowest number of sequences) to avoid bias due to uneven read numbers across samples.The pyrosequence reads have been submitted to the NCBI Short Read Archive under accession number SRP049034.

qPCR analysis. For estimation of the 16S rRNA gene abundance in the pyrosequenced samples, amplifications of each sample of extracted DNA were performed with general bacterial primers (Table 2). The reaction mix comprised 10 μl Power SYBR green PCR master mix (Applied Biosystems), 2 μl 10 μM primers, and precisely 25 ng DNA template in a 20-μl volume, with reagent-only samples as negative controls. Amplifications were conducted in a C1000 thermal cycler (Bio-Rad) with the following thermal profile: 95°C for 5 min, 40 amplification cycles of 95°C for 15 s, 55.2°C for 30 s, and 60°C for 30 s, and a dissociation cycle of 95°C for 15 s, 60°C for 15 s, and then brought back to 95°C. The mean threshold cycle (C_T) values of three technical replicates per pyrosequence sample were calculated.

The same qPCR protocol was used to quantify the abundance of individual bacteria in the gnotobiotic flies, but species-specific 16S rRNA gene bacterial primers were used and the *Drosophila gapdh* gene was used for normalization to the host (Table 2); modification of the thermal cycle to include a 54°C-to-57°C gradient (see melting temperature [T_m] data in Table 2) for 30 s in the amplification step. The dissociation curve confirmed that every reaction mixture yielded a single PCR product with the predicted T_m. To determine the relative abundance of each bacterial species, we first corrected for primer efficiencies by using the following formula: $C_{T(\text{corrected})} = C_{T(\text{measured})} \times [\log(E_{\text{measured}})/\log(E_{\text{optimum}})]$, where E denotes the primer efficiency (optimum = 2). The mean C_T values of technical duplicates were determined and used to obtain $\log_2$ fold differences, as follows: $\Delta C_T = C_{T(\text{bacterium-specific primer})} - C_{T(\text{reference gene})}$. Values were transformed to the linear scale, and relative abundances of bacterial species were calculated.

Enumeration of CFU. Samples of food and *Drosophila* (for details of the methods used for sampling, see the supplemental material) were homogenized in MMRS medium and plated onto duplicate plates of MMRS agar with a WASP-2 instrument (Microbiology International). The plates were either supplemented with 10 μg ampicillin ml^{-1} or incubated in a CO_2 atmosphere, for the selective growth of *Acetobacter* and *Lactobacillus*, respectively (20). CFU were counted with the Protocol 3 colony counter (Microbiology International). Any plates that bore colonies which did not conform to the morphology of *Acetobacter* or *Lactobacillus* (small, tan colonies and white colonies, respectively, as described previously [22]) were discarded.

Experimental designs. To investigate the relationship between the composition of the microbiota associated with *Drosophila* flies and their food, two replicate vials of nonsterile food were inoculated with 20 unmanipulated eggs or 20 dechorionated eggs, with two egg-free vials as control, and maintained in the fly chamber to day 16, when the *Drosophila*

TABLE 1 Primers used for pyrosequencing[a]

Source	Sample no.	Forward primer	Reverse primer
Food, day 0	Vial 1	CGTATCGCCTCCCTCGCGCCATCAGCGTGTCTCTAAGAGTTT GATCMTGGCTCAG	CTATGCGCCTTGCCAGCCCGCTCAGTGC TGCCTCCCGTAGGAGT
	Vial 2	CGTATCGCCTCCCTCGCGCCATCAGCTCGCGTGTCAGAGTTT GATCMTGGCTCAG	
	Vial 3	CGTATCGCCTCCCTCGCGCCATCAGACGAGTGCGTAGAGTTT GATCMTGGCTCAG	
	Vial 4	CGTATCGCCTCCCTCGCGCCATCAGAGACGCACTCAGAGTTT GATCMTGGCTCAG	
	Vial 5	CGTATCGCCTCCCTCGCGCCATCAGTGTACTACTCAGAGTTT GATCMTGGCTCAG	
	Vial 6	CGTATCGCCTCCCTCGCGCCATCAGACGACTACAGAGAGTTT GATCMTGGCTCAG	
Food, day 16	Vial 1	CGTATCGCCTCCCTCGCGCCATCAGTCTCTATGCGAGAGTTT GATCMTGGCTCAG	
	Vial 2	CGTATCGCCTCCCTCGCGCCATCAGTGATACGTCTAGAGTTT GATCMTGGCTCAG	
	Vial 3	CGTATCGCCTCCCTCGCGCCATCAGAGCACTGTAGAGAGTTT GATCMTGGCTCAG	
	Vial 4	CGTATCGCCTCCCTCGCGCCATCAGATATCGCGAGAGAGTTT GATCMTGGCTCAG	
	Vial 5	CGTATCGCCTCCCTCGCGCCATCAGTACGAGTATGAGAGTTT GATCMTGGCTCAG	
	Vial 6	CGTATCGCCTCCCTCGCGCCATCAGTACTCTCGTGAGAGTTT GATCMTGGCTCAG	
Drosophila	Eggs	CGTATCGCCTCCCTCGCGCCATCAGTCGTCGCTCGAGAGTTT GATCMTGGCTCAG	
	Vial 1	CGTATCGCCTCCCTCGCGCCATCAGTCACGTACTAAGAGTTT GATCMTGGCTCAG	
	Vial 2	CGTATCGCCTCCCTCGCGCCATCAGCGTCTAGTACAGAGTTT GATCMTGGCTCAG	
	Vial 3	CGTATCGCCTCCCTCGCGCCATCAGCATAGTAGTGAGAGTTT GATCMTGGCTCAG	
	Vial 4	CGTATCGCCTCCCTCGCGCCATCAGATACGACGTAAGAGTTT GATCMTGGCTCAG	
Environment	Sample 1	CGTATCGCCTCCCTCGCGCCATCAGACTACTATGTAGAGTTT GATCMTGGCTCAG	
	Sample 2	CGTATCGCCTCCCTCGCGCCATCAGACGCGAGTATAGAGTTT GATCMTGGCTCAG	
Reagent-only control		CGTATCGCCTCCCTCGCGCCATCAGACATACGCGTAGAGTTT GATCMTGGCTCAG	

[a] The general primers PrimerA_MID_27F and PrimerB_338R were tagged with different MIDs to produce the indicated primers.

were 5- to 6-day-old adults. The following samples were collected for pyrosequencing: one sample of unmanipulated eggs on day 0, one surface sample of food from each vial on both day 0 (pre-egg inoculation) and day 16, one sample of adult flies from each vial previously inoculated with eggs on day 16 (pools of 3 males and 3 females), and two environmental samples (swabs of the incubator in which the flies were raised) placed in PBS buffer on day 16. The data are displayed for a single experiment because the composition of the bacteria in nonsterile food and flies varies over the time scale of months (16, 21; unpublished data), confounding attempts to combine results from experiments conducted on different occasions.

Subsequent experiments used gnotobiotic flies raised on medium containing cultured bacteria (*Acetobacter pomorum*, *A. tropicalis*, *Lactobacillus brevis*, and *L. plantarum*) previously isolated from *Drosophila* guts (22) to investigate the bacterial abundance and community composition in the food and *Drosophila* flies. An additional bacterium, *L. fructivorans*, used in our previous research (20), was not included because its low growth rate precluded quantification of CFU in the presence of the other bacteria (20, 22).

To determine the density of bacteria in the surface and core samples of the food, 10 vials of sterile food were seeded with the four-species inoculum, and 30 dechorionated eggs were aseptically added to half of these vials. A surface sample and core sample were taken from 5 vials with *Drosophila* (+D) and 5 *Drosophila*-free (no-D) vials at day 16 (5- to 6-day-old adult flies).

To quantify the impact of *Drosophila* density on bacterial communities in the food, 10 replicate vials were inoculated with 0, 10, 30, or 60 dechorionated eggs, and the CFU of *Acetobacter* and *Lactobacillus* in surface food samples were determined on day 16. In parallel, one sample of 5 male flies was collected from each of the 10 vials in which 30 eggs had been administered, for parallel quantification of bacteria by CFU and qPCR analysis.

The final experiment determined the impact of *Drosophila* and *Aceto-*

TABLE 2 Primers used for qPCR

Target	Orientation	Primer sequence (5′–3′)	Efficiency	T_m[a]
Acetobacter pomorum[b]	Forward	CTAGATGTTGGGTGACTTAGTCA	1.79	54.6
	Reverse	CGGGAAACAAACATCTCTGCTTG		
Acetobacter tropicalis	Forward	GGACAACTTAGTTGTTCAGTGTC	1.83	55.9
	Reverse	GGACACAGCCTACACATACAAG		
Lactobacillus brevis	Forward	GACGTGCTTGCACTGATTTC	1.98	54.2
	Reverse	CCGAAGCCACCTTTCAAAC		
Lactobacillus plantarum[b]	Forward	CGAACGAACTCTGGTATTGATTG	1.93	56.8
	Reverse	ACCATGCGGTCCAAGTTG		
D. melanogaster GAPDH	Forward	TAAATTCGACTCGACTCACGGT	1.92	57.0
	Reverse	CTCCACCACATACTCGGCTC		
General 16S rRNA (341F + 534R)	Forward	CCTACGGGAGGCAGCAG	1.96	55.2
	Reverse	ATTACCGCGGCTGCTGG		

[a] The optimal annealing temperature was determined via gradient qPCR in order to achieve the highest primer efficiency.
[b] The Ribosomal Database Project (46) probe match indicated that the *A. pomorum* primers may also amplify strains of the closely related species *A. pasteurianus* and the *L. plantarum* primers may also amplify strains of the closely related *L. pentosus*.

bacter on the fate of *Lactobacillus* in the food by a two-by-two factorial design (with/without *Drosophila* and *Acetobacter*) with 10 replicate vials per treatment. Thirty dechorionated eggs were added to 20 vials, leaving the other 20 vials *Drosophila*-free. Ten vials in each of the two *Drosophila* treatments were then inoculated with the two *Lactobacillus* species, and the other 10 vials were inoculated with both *Lactobacillus* and *Acetobacter* species (4 species in total). The food was sampled 16 days later for *Lactobacillus* CFU.

Statistical analysis. Parametric statistical tests were applied to normally distributed data sets with equal variance, as assessed by the Anderson-Darling test and Bartlett's test, respectively. The CFU data set for *Lactobacillus* in vials containing 60 *Drosophila* eggs and all *Acetobacter* data sets met these criteria after logarithmic transformation. The *Drosophila* survival data and all other *Lactobacillus* data sets were analyzed by nonparametric tests. Tukey's test was used for *post hoc* testing after an analysis of variance.

RESULTS

The source of bacteria in *Drosophila*. The food vial is the habitat for laboratory cultures of *Drosophila*. To elucidate the contribution of microorganisms to the fly microbiota from the food, the environment (i.e., the fly incubator), and the eggshell, we determined the bacterial communities in 6 replicate vials of food by pyrosequencing 16S rRNA gene amplicons and then added either unmanipulated or dechorionated (i.e., germfree) eggs to two of the vials, leaving two vials *Drosophila*-free. Sixteen days later, we redetermined the bacteria in the 6 vials. We also assayed unmanipulated eggs on day 0 and environmental samples and the *Drosophila* flies at 5 to 6 days posteclosion, i.e., on day 16.

The three sources of bacteria (food, environment, and unmanipulated *Drosophila* eggshells) yielded 535 OTUs, but only 11 were detected in all three sources (Fig. 1A; see also Tables S1 and S2 in the supplemental material). *Acetobacter* and *Lactobacillus* species accounted for the majority of the *Drosophila* egg microbiota (>89% of reads) but <1% of the reads in the fly-free food samples prior to adding the *Drosophila* eggs (i.e., vials 1 to 4 on day 0) and 7 to 8% of the environmental samples. The fly-free food samples were dominated by various *Bacilli* (but not *Lactobacillus*) and *Moraxellaceae* (*Gammaproteobacteria*). The environmental samples comprised mainly *Gammaproteobacteria*, most notably *Xanthomonadales*, which constituted >50% of the reads, as well as *Burkholderiales* (see Table S1).

The microbiota of flies from unmanipulated eggs was dominated by three species of *Acetobacter* (*A.aceti*, *A. malorum/tropicalis*, and *A. pomorum/pasteurianus*), which accounted for >95% of the reads (Fig. 1B). As illustrated in the weighted principal coordinates plot (Fig. 1C), the microbiota in flies from the unmanipulated eggs generally resembled the egg microbiota. Although these flies also possessed some OTUs also present in the fly-free food on day 0 (99/244 OTUs), the environment (4/244 OTUs), or both (19/244 OTUs), these taxa collectively accounted for <6% of the total reads in the fly samples. Overall, the mean weighted UniFrac distance between the bacterial communities in these flies and the eggs was 0.09, and that between the flies and food on day 0 was 0.32 (see Table S3 in the supplemental material). These findings indicate that the microbiota of *Drosophila* is derived predominantly from the microbiota associated with eggs in our laboratory system. Reinforcing this conclusion, we have consistently failed to isolate *Acetobacter* from food vials that have not been exposed to *Drosophila* (unpublished data). Furthermore, the bacterial communities in the food bearing these flies shifted, between day 0 and day 16, to closely match the bacterial composition in the flies (UniFrac distance 0.02 to 0.03) (see Table S3).

The results for the vials administered unmanipulated eggs demonstrated the transmission of bacteria associated with the eggs to the next generation of flies, but they did not discriminate whether or how microbiota-independent traits of *Drosophila* may contribute to the change in microbiota composition in the food between day 0 and day 16. This issue was addressed by the other treatments: if the composition of the microbiota were influenced by the *Drosophila*, the microbiota would be predicted to differ between *Drosophila*-free vials and vials administered dechorionated eggs (i.e., *Drosophila* but no microorganisms). The *Drosophila* reared from dechorionated eggs bore a microbiota of higher diversity than those from unmanipulated eggs (see Table S2 in the supplemental material) that included various bacterial taxa, e.g.,

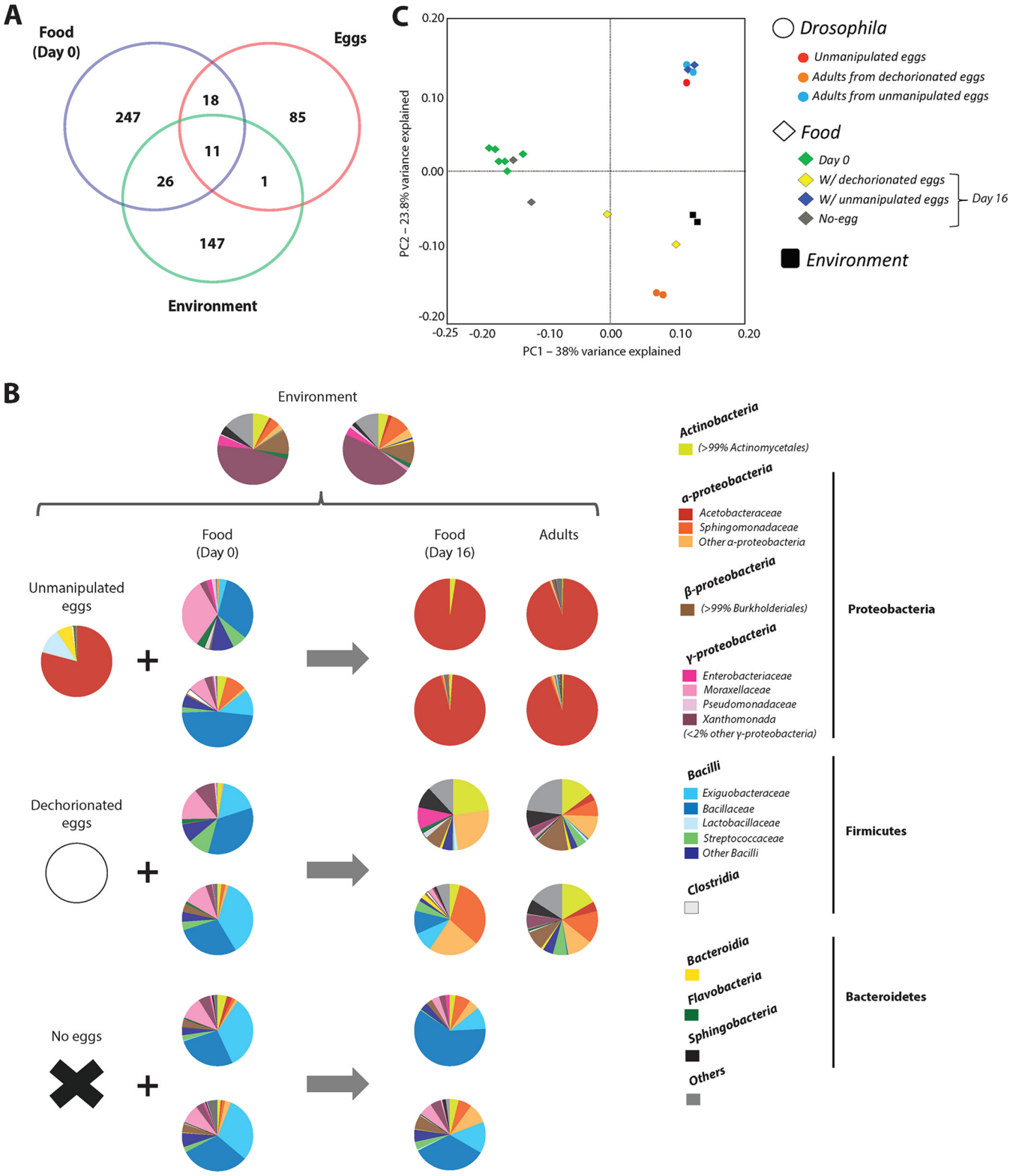

FIG 1 Bacterial communities in *Drosophila* and food. (A) Shared and unique OTUs among the sources: unmanipulated eggs, food on day 0, and environmental samples (swabs from the fly incubator). (B) Relative abundance of the major genera and phyla, displayed as pie charts of proportional abundances of bacterial taxa in food, flies, and environmental samples. (C) Relationship between bacterial community composition, displayed as a weighted Unifrac PCoA, among the food samples (day 0 and day 16) from vials administered eggs on day 0 (with no-egg vials as negative controls), fly samples (day 16) derived from unmanipulated or dechorionated eggs, and environmental samples (described above).

Actinobacteria, *Sphingomonas*, and *Burkholderia* (Fig. 1), of low abundance in the fly-free food. Furthermore, the microbiota in the food differed between the *Drosophila*-free vials and the vials bearing *Drosophila* flies from dechorionated eggs. Specifically, the relative abundance of reads assigned to *Bacilli* in the food at day 0 (52 to 73% abundance) was sustained with the no-egg treatment at day 16, but much diminished (6% and 26%) in the presence of *Drosophila* flies from dechorionated eggs, being replaced by bacterial communities similar to that in the flies (Fig. 1B). Overall, the composition of the food microbiota at day 16 containing flies raised from dechorionated eggs was similar to that of the flies in these vials, whereas the bacterial communities in the *Drosophila*-free vials underwent little change (the UniFrac distance between the food on day 0 and day 16 was 0.29 and 0.33 in vials administered dechorionated eggs and 0.13 and 0.16 in the *Drosophila*-free vials).

The pyrosequence analysis was conducted on 16S rRNA gene amplicons generated with total DNA as the template, and consequently the absolute abundance of the bacteria identified in the various samples is unknown. As a complementary analysis, the abundance of the bacterial 16S rRNA gene in the samples was quantified by qPCR using general 16S rRNA gene primers with the same amount of template DNA per sample (see Table S4 in the supplemental material). The C_T values for fly samples from dechorionated eggs and unmanipulated eggs (mean values of 17.04 and 16.21, respectively) differed by less than one unit, confirming that the flies in the two treatments support bacterial communities of broadly equivalent magnitudes.

The two key conclusions from these experiments are that the composition of the microbiota associated with food is altered by the presence of *Drosophila*, and that the bacterial communities in the food supporting a *Drosophila* culture are very similar in composition to the communities in the flies. However, pyrosequence data provided no information on the traits of the various bacteria contributing amplicons (e.g., viability, capacity for population growth, etc.). To investigate the processes by which *Drosophila* influence the bacterial content of the food, we used flies colonized with a standardized community of four bacterial species, *Acetobacter pomorum*, *A. tropicalis*, *Lactobacillus brevis*, and *L. plantarum*. This approach enabled absolute quantification of the population size of living bacterial cells (as CFU) and excluded among-experiment variation caused by stochastic variation in microbiota composition.

The relationship between bacteria in the food and *Drosophila*. Flies were raised from dechorionated eggs aseptically transferred to autoclaved food that had been administered the standardized community of *Acetobacter* and *Lactobacillus*. Contrary to our previous research, in which *Drosophila* eggs incubated with these bacteria yielded adult flies containing similar densities of *Acetobacter* and *Lactobacillus* (20), the bacteria associated with the resultant 5- to 6-day-old flies were dominated by *Acetobacter*. In an analysis of flies from 10 replicate vials, the median number of *Acetobacter* CFU per fly was 6×10^3 for males and 16.9×10^3 for females, 2 orders of magnitude greater than the equivalent values for *Lactobacillus* (0.08×10^3 and 0.16×10^3 per fly, respectively). Based on parallel quantification of male fly samples from 10 replicate vials by qPCR of 16S rRNA gene sequence using species-specific primers, the median percent contribution of the four bacterial species (ranges in parentheses) were as follows: *A. pomorum*, 29.9% (9.2 to 46.2%); *A. tropicalis*, 69.9% (53.7 to 90.3%); *L. brevis*, 2.2% (1.2 to 3.6%); *L. plantarum*, 0.15% (0.05 to 0.6%), providing confirmation that *Acetobacter* and *Lactobacillus* differed in abundance in the flies and not in culturability. These results provided the opportunity to establish the processes by which the different fates of *Acetobacter* and *Lactobacillus* species are determined, as well as to investigate possible reasons for the discrepancy with our previously published results.

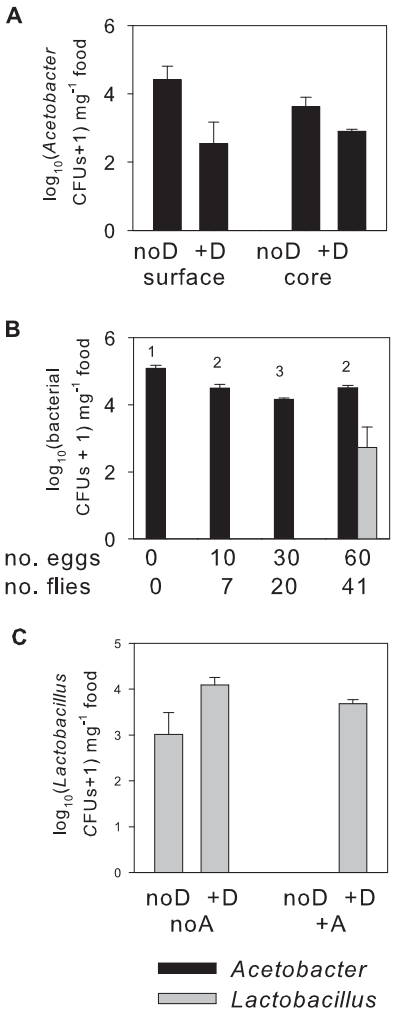

FIG 2 Impact of *Drosophila* on the abundance of bacteria in food. Vials were inoculated on day 0 with four bacterial species (*A. pomorum*, *A. tropicalis*, *L. brevis*, and *L. plantarum*) in all treatment groups except for "noAce" (no *Acetobacter* [panel C]), which were inoculated with *L. brevis* and *L. plantarum* only. Treatment "noD" consisted of *Drosophila*-free vials, and the "+D" group consisted of dechorionated *Drosophila* eggs added at day 0. (A) Density of *Acetobacter* spp. in core samples and surface samples taken at day 16 (+D vials bore 30 eggs). Values are means ± SE; data from 4 replicate experiments are shown. (B) Density of *Acetobacter* spp. and *Lactobacillus* spp. after 16 days on food supplemented with different numbers of dechorionated *Drosophila* eggs (means ± SE of 10 replicates are shown). The mean number of live flies on day 16 is indicated. Mean values of *Acetobacter* with different superscript numbers (1 to 3) differed significantly based on Tukey's *post hoc* test. (C) Density of *Lactobacillus* spp. after 16 days on food, following inoculation without (noA) or with (+A) *Acetobacter* spp. in noD and +D vials. Means ± SE for 10 replicate vials are shown.

First, we investigated the impact of *Drosophila* on the bacterial populations in the food (Fig. 2A). At day 16, all the bacterial colonies derived from both core samples (representative of the total

bacterial content of the food) and surface samples (representative of the food consumed by adult flies) could be assigned to *Acetobacter*, confirming the link between bacteria in the flies and food and demonstrating that the dominance of *Acetobacter* was not *Drosophila* dependent. The abundance of *Acetobacter* at day 16 was depressed in vials containing *Drosophila* ($F_{1,16} = 10.53$, $P = 0.005$). For the core samples, which provided an estimate of the total *Acetobacter* content of the food in the vials, the mean *Acetobacter* density was significantly elevated relative to the starting inoculum ($t_4 = 7.23$ and 4.02 for vials with and without *Drosophila*, respectively; $P < 0.025$). Supplementary analyses to determine the timing of the different fates of *Acetobacter* and *Lactobacillus* showed that *Acetobacter* persisted at least 15 days, but that no *Lactobacillus* CFU were recovered by day 10 for any vial containing *Drosophila* and day 11 for the *Drosophila*-free vials (see Fig. S1 in the supplemental material).

Turning to the discrepancy between the abundance of *Lactobacillus* in this study and our previous research, we noted one difference in the protocol: this study added 30 eggs to each vial, but more eggs were used in our previous research (although the numbers were not quantified precisely). Pilot experiments with different numbers of eggs (0 to >100) recovered *Lactobacillus* from the food in vials administered at least 50 eggs, but not fewer eggs, suggesting that high *Drosophila* densities promote *Lactobacillus*. As a definitive test of this hypothesis, we quantified the effect of egg number (0, 10, 30, and 60 sterile eggs in 10 replicate vials) on the bacterial complement of the food. Fly survivorship did not vary with egg density, with all treatments yielding ca. 70% live flies from the eggs (Kruskall Wallis analysis, H2 = 0.23, $P = 0.89$) (Fig. 2B). The abundance of *Acetobacter* in surface samples of the food varied significantly across the four classes of administered egg number ($F_{3,36} = 20.42$, $P < 0.001$) and was significantly depressed in vials containing *Drosophila* relative to vials without *Drosophila* (Fig. 2B). *Lactobacillus* was absent from all vials administered 0, 10, or 30 eggs. Exceptionally, the vials with 60 *Drosophila* eggs (mean of 41 flies) supported *Lactobacillus*, at a mean density of 539 CFU mg of food^{-1}, representing 1.6% of the 32,200 CFU of *Acetobacter* mg^{-1} in the same vials (values back-transformed from logarithmic means). Supplementary experiments demonstrated that this effect was highly repeatable.

To investigate the basis of the positive effect of high *Drosophila* densities on *Lactobacillus* abundance in the food, the food was inoculated with *Lactobacillus* in the presence/absence of *Acetobacter* and *Drosophila* eggs at densities that yielded 43.2 ± 2.90 (mean ± standard error; $n = 10$) adult flies on day 16. *Lactobacillus* was detected in vials coinoculated with *Acetobacter* only when *Drosophila* flies were also present, and *Lactobacillus* attained a mean density of 4.8×10^3 CFU mg of food^{-1} (back-transformed from logarithmic mean in Fig. 2C). This result is consistent with the data in Fig. 2B, although the *Lactobacillus* abundance in the +*Drosophila* treatment differed by an order of magnitude between the two experiments. (The reasons for the among-experiment variation in *Lactobacillus* abundance in vials bearing ≥40 *Drosophila* have not been investigated.) For the *Acetobacter*-free treatments, *Lactobacillus* was recovered from all vials, with a 10-fold-greater mean density in vials with *Drosophila* (1.23×10^4 CFU mg of food^{-1}) than without *Drosophila* (1.0×10^3 CFU mg of food^{-1}) (mean values back-transformed from logarithmic values in Fig. 2C), and this effect was statistically significant (log-transformed data: $t_{18} = 2.24$, $P < 0.05$). These results indicated that *Lactobacillus* proliferation is supported by the food and promoted further by *Drosophila*. The significantly lower abundance of *Lactobacillus* in +*Drosophila* vials containing *Acetobacter* than without *Acetobacter* ($t_{18} = 2.28$, $P < 0.05$) indicates that *Drosophila* flies did not fully overcome the negative effect of the *Lactobacillus/Acetobacter* antagonism on *Lactobacillus*.

DISCUSSION

A key finding of our pyrosequencing analysis is that the bacterial communities in the flies and the food in laboratory cultures of *Drosophila* are closely matched. The difference between the bacterial community in *Drosophila*-free vials and vials administered bacteria-free *Drosophila* (Fig. 1) suggests that the *Drosophila* play a key role in this pattern; it is not readily compatible with the alternative scenario, that the microbiota in the gut is a passive readout of the microbiota in the fly-free food to which the *Drosophila* are introduced. The nature of the microbiota-independent effects of *Drosophila* on the bacterial populations in the food remains to be determined but may include changes to the physical structure of the food by feeding activity and larval tunneling, or alterations to the chemical constitution of the food by release of enzymes and other bioactive compounds in regurgitant, saliva, and fecal material. Our study additionally shows that alternative *Drosophila*-dependent bacterial communities can be assembled on a single diet formulation (Fig. 1), confirming evidence from other studies that *Drosophila* can associate with many different bacteria (16, 21, 28). These conclusions should, however, be tempered by the widely-recognized limitations of amplicon analysis of microbial communities, including variation in the extraction and amplification efficiencies of different bacterial taxa, the inability of this method to discriminate between DNA from live and dead bacterial cells, and the artifactual inclusion of contaminating sequences, especially from reagents (29–31). In our experiments, we made every attempt to minimize the limitations brought by these caveats, including the use of an optimized DNA extraction protocol and correction for sequences recovered from reagent-only controls (see Materials and Methods).

Multiple factors can shape the composition of the matching bacterial communities in the *Drosophila* flies and the food. One significant factor is the compatibility of the bacteria with both the *Drosophila* gut and the food; this factor likely contributes to the variation in the gut microbiota composition with diet in *Drosophila* (16, 17) and other animals with open symbioses (32–34). Bacteria-bacteria interactions are also important, as illustrated by the replacement of the bacterial communities in fly-free food by bacteria of the genus *Acetobacter* introduced with the *Drosophila* eggs (Fig. 1) and the suppression of *Lactobacillus* by *Acetobacter* in food bearing few or no *Drosophila* (Fig. 2). However, *Drosophila* can influence the outcome of these among-bacterial interactions, as illustrated by the recovery of *Lactobacillus* in vials coinoculated with *Acetobacter* at high densities of *Drosophila*. The basis of the density-dependent suppression by *Drosophila* of the negative effect of the *Acetobacter/Lactobacillus* antagonism on *Lactobacillus* abundance may be multifaceted. For example, *Drosophila* feces or other products may provide nutrients utilizable by *Lactobacillus* but not *Acetobacter*, and the feeding activity and movement of *Drosophila* may disrupt the otherwise-coherent biofilm formed by *Acetobacter* on the surface of the food (unpublished observations), potentially reducing *Acetobacter* competitiveness.

The relationship between the bacterial populations in the *Dro-*

sophila gut and the food cannot be understood fully without considering the costs and benefits of the association with the animal and microbial partners. The natural diet of *Drosophila* is rotting fruit and the microorganisms (yeasts and bacteria) that contribute to fruit degradation. Available evidence suggests that *Drosophila* benefits from the relationship by gaining access to nutrients, especially amino-N, lipids, and B vitamins, from the microorganisms (35–39). This study reveals that the association can be costly to the bacterial partner. Specifically, the total abundance of *Acetobacter* in vials bearing *Drosophila* was depressed relative to that in fly-free vials (Fig. 2A). Potentially outweighing this cost is the likely benefit to the microbiota of host-mediated dispersal, achieved by the voiding of viable microbial cells in the feces (14, 15, 19, 40). The magnitude of the costs and benefits to the bacteria of associating with *Drosophila* is expected to vary with conditions and traits of the different bacteria. For example, bacteria with relatively long retention times in the *Drosophila* gut are likely to be more favored in wild *Drosophila* populations than in laboratory cultures, where the continual access of flies to food is predicted to result in relaxed selection on the bacteria to persist for extended periods in the gut.

The crucial outstanding question is the extent of coevolutionary interactions between the partners in open systems, as exemplified by the relationship between *Drosophila* and its gut microbiota. For example, the digestive and immunological functions of the insect gut may be calibrated for bacterial survival and shedding rates that optimize dispersal of beneficial microorganisms to microsites utilized by the insect host and its progeny, while the living cells of beneficial microorganisms may display adaptations that promote the release of nutrients valuable to the host (B vitamins and amino-N) and mediate putrefaction of fruit at rates appropriate to preadult development rates of the insect. The apparent absence of a taxonomically defined core microbiota in *Drosophila* (21) suggests that such coevolutionary interactions would be diffuse, involving guilds of microorganisms interacting with multiple animal taxa associated with a diversity of fruits. In addition, *Drosophila*-associated yeasts have been proposed to modify the fruit habitat to the benefit of *Drosophila*, a process interpreted as niche construction, and release volatiles that are utilized as foraging and oviposition cues by the insect host (41). Bacterial partners may also contribute to these effects.

In conclusion, multiple instances are known of animals that control the composition of microbial communities in the external environment, but these systems predominantly involve external habitats created and managed by complex behavioral traits of the animal. Examples include the fungal nests maintained by attine ants and macrotermitine termites and the microbes that line the galleries of ambrosia beetles and brood chambers of crabronid wasps (42–44), as well as the production of alcohol, bread, yogurts, and fermented vegetables, etc., by humans. This study on the relationship between *Drosophila* and its gut microbiota demonstrates that relatively nonspecific associations that apparently lack sophisticated behavioral mechanisms for controls over the microbiota can similarly play a role in shaping microbiota communities in the wider environment. Priorities for future research include quantitative analysis of the dynamics of bacterial transfer between the host and external environment (45) and investigation of how the principles of these interactions, identified under defined conditions on standardized food substrates, apply to the spatiotemporally complex habitat of a rotting fruit under natural conditions.

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